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Confirmed Presence of Parahepatospora carcini in Carcinus maenas Population of Maine

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CONFIRMED PRESENCE OF *PARAHEPATOSPORA CARCINI* IN *CARCINUS*
MAENAS POPULATION OF MAINE

by

Brittany Torchia

A Thesis Submitted to Partial Fulfillment
of the Requirements for a Degree with Honors
(Marine Science)

The Honors College

The University of Maine

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Advisory Committee

Ian Bricknell, Professor of Aquaculture, Advisor

Timothy Bowden, Associate Professor of Aquaculture

Kristina Cammen, Assistant Professor of Marine Mammal Science

Mark Haggerty, Rezendes Professor for Civic Engagement in the Honors College

Heather Hamlin, Associate Professor of Marine Biology and Aquaculture

ABSTRACT

Carcinus maenas (European green crab) is an invasive species that made its way to North American waters in the 1800s on European trading ships. As an invasive species they cause problems by competing with native species for the resources within their shared ecosystem. They can also introduce pathogens that can infect and wreak havoc on native populations. In Nova Scotia, Bojko et al. (2017) found the pathogen *Parahepatospora carcini*, a clade IV microsporidian parasite found in the hepatopancreas of aquatic arthropods, in *C. maenas*. Because this case was located within close range of Maine waters, the objective of this study was to determine if Maine's green crab population was also infected. In a sample of 500 green crabs, seven were found to be infected, resulting in a 1.4% prevalence of *P. carcini* within the *C. maenas* population of Maine. The seven infected crabs had a smaller range and average size than the entire sample, suggesting that *C. maenas* might not survive long after infection or that growth is stunted due to lack of energy stores. *P. carcini* prevalence in this sample peaked just before mid-July, then tapered off later in the month indicating that there could be a seasonal component to the parasite's life cycle. However, a larger sample size and a year round collection would be needed to determine if these factors have a significant correlation with *P. carcini* prevalence. Sample location, crab sex, and organ color had no correlation with infection prevalence. PCR resulted in a pathogen band at 939 bp in all seven samples, which was determined to be from a microsporidian DNA by sequencing an excised and purified band. Histology was used to confirm *P. carcini* infection in all seven samples with both H&E and trichrome staining techniques.

All data and results confirm that *P. carcini* is present in the green crab population of Maine, the first place it has been recorded in the United States.

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INTRODUCTION

Carcinus maenas is perhaps one of the most infamous invasive species in coastal Maine waters. They were first brought to North America in the 1800s on ships sailing across the Atlantic from Europe to America. It is believed that they were brought over in the ballast water of these ships, then found the New England coast to be a suitable habitat and began establishing a population. Their phenotypic plasticity, wide temperature and salinity tolerance, and omnivorous diet made *C. maenas* especially hearty and successful (Young & Elliott, 2020). Due to rising water temperatures the range of these pests has expanded over the years (Figure 1) and they can now be found all over the northeastern coastline from Delaware to Nova Scotia (Trussel, 2000). As an invasive species, green crabs wreak havoc on ecosystems by competing with natural populations for limited resources, such as food and shelter, without any natural predators to limit their numbers.

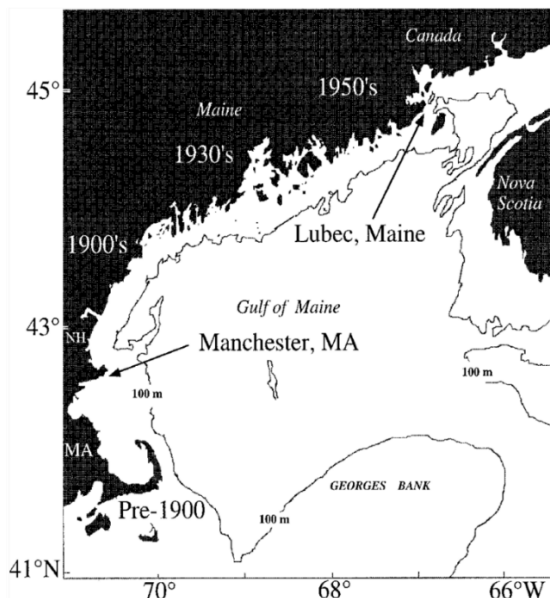


Figure 1. Map of the Gulf of Maine showing the northward progress of the *Carcinus maenas* biogeographic range expansion from 1900 to 1950 (based on Scattergood, 1952; Vermeij, 1978; (Trussel 2000).

Within the state of Maine alone, *C. maenas* has destroyed entire ecosystems and caused the decline of an economically important fishery. Between the years 2012 and 2013 over half of the eelgrass (*Zostera marina*) cover vanished from Casco Bay. During this same time period *C. maenas* population increased dramatically, making researchers suspicious that they were the culprit. Sure enough, when a trial was performed examining the survival of eelgrass grown both inside and outside green crab enclosures, *Z. marina* had significantly lower survival rates when green crabs could access it. This proved that the bioturbation activities of *C. maenas* were the leading cause of the ecosystem's eelgrass loss (Neckles, 2015). The softshell clam fishery is Maine's third largest fishery in terms of value (Maine Sea Grant) and produces 60% of the country's soft-shell clams. In 2017, the industry was valued at \$36 million, but since the 1980s statewide landings have declined by 75% (Downeast Institute). This decline in stock has been proven to be caused by increased predation from green crabs that are being saved from freezing during cold winters by global warming (Congleton et al. 2006).

Invasive species like *C. maenas* can also harm native species by introducing new pathogens into the native community (Bojko 2017). This can occur very easily if an infected individual carrying the pathogen immigrates into a new population with suitable hosts. Many of these pathogens are capable of spreading among organisms that are similar to the one carrying it. For example, if a crustacean like the green crab is carrying a pathogen that infects the hepatopancreas, then that pathogen could infect other crustaceans with a hepatopancreas, such as the American Lobster (*Homarus americanus*). A pathogen like this could reduce the population size of the native species or make them

unsafe to consume. This would be cause for concern in a state like Maine where the economy is heavily reliant on fishing industries.

One such pathogen that is carried by green crabs is *Parahepatospora carcini*, a species of microsporidia that is in a sister clade to the fungi kingdom (Bojko 2017), although it is unknown if it originated in the invasive or natural range of *C. maenas*. Microsporidians are parasitic pathogens that are unicellular and form spores inside host cells of almost all animals ranging from single celled protists to vertebrates (Pan, G. 2018), making them quite common. Almost half of the known genera of microsporidians affect aquatic organisms and some even have the potential for zoonotic transfer (Stentiford 2013).

P. carcini specifically affects the hepatopancreas in aquatic arthropods, damaging the tubules of the hepatopancreas (Stentiford 2013). As described and depicted in figure 2 by Bojko et al. (2017), it does this by entering the cell as a uninucleate meront at the periphery of the interfacial membrane. Once inside the cell, *P. carcini* unikaryotic meronts divide without cytokinesis, creating diplokaryotic meronts. The cell cytoplasm then darkens before more nuclear divisions occur to create multinucleate meronts. These cells invaginate and elongate and the plasmodial wall thickens to prepare for primary spore organelle formation. The pre-sporoblasts can then separate from the sporont plasmodium to form sporoblasts containing a single nucleus. As the spores develop, they migrate to the center of the cell, nearest the lumen. The infection will progress and more spores will form and occupy the cell until eventually the cells are sloughed from the basal membrane, releasing the spores into the lumen and damaging the structure of the cell (Bojko et al., 2017). This compromised structure inhibits the function of the cell, and

when enough cells have been infected it will negatively affect the function of the organ as a whole. The hepatopancreas is a digestive organ that combines the functions of the liver and pancreas in mammals. It can both synthesize and secrete digestive enzymes and absorb and store nutrients. This is especially important during molting, starvation, and reproduction phases when it is necessary to store large amounts of energy within the hepatopancreas in the form of lipids (Wang et al. 2014). Damage to this organ would lead to problems obtaining and storing the necessary nutrients from food to generate energy for metabolism.

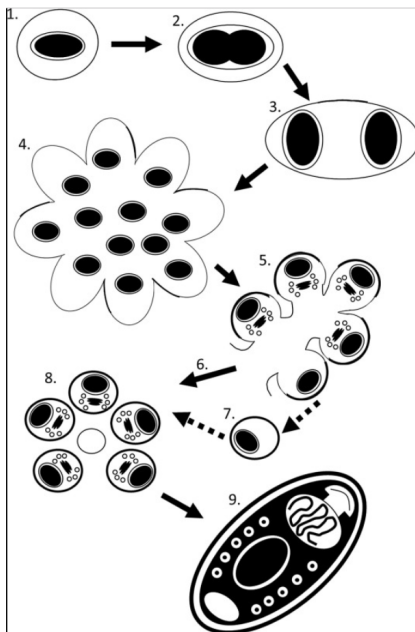


Figure 2. Predicted lifecycle of *Parahepatospora carcini* (Bojko 2017).

Within its invasive range in the United States, very few parasites exist to keep *C. maenas* populations in check that would be present in its natural range. For example, in Europe approximately 16% of green crabs are infected with the Rhizocephalan parasite *Sacculina carcini*, but it has not been found within U.S. populations (Young & Elliott, 2020). Therefore, it is especially important to study new pathogens as they emerge within the invasive range of *C. maenas*. Although its origin is unknown, *P. carcini* has been

found by Bojko et al. (2017) in Nova Scotia's green crab population. Because Nova Scotia's coastline neighbors Maine's, the goal of this study was to determine if *P. carcini* is present in Maine's green crab population and if so, what its prevalence is.

MATERIALS AND METHODS

Sample Collection

A total of 500 *Carcinus maenus* were sampled during the months of June and July, 2019. 250 were collected from Kettle Cove State Park in Cape Elizabeth, Maine and another 250 were collected from Roque Bluffs State Park in Roque Bluffs, Maine. Both sampling locations are shown on the map below (Figure 3.). A sample size of 500 animals was chosen due to the low prevalence rate of 0.7% found by Bojko et al. (2017). Animals were collected both by hand at low tide and using a salmon-baited crab pot. Once collection was finished for the day, the crabs were transported in a damp cooler back to the Bricknell lab at the University of Maine, Orono campus. They were kept in the damp cooler overnight, or until they could be processed later that day. The crabs were euthanized with an injection of super saturated potassium chloride then carapace width was measured and sex was recorded. Each animal was then necropsied and the hepatopancreas was dissected after organ color was recorded. The hepatopancreas was halved and split into two solutions: 95% ethanol for DNA extraction and 10% formal seawater for histology.



Figure 3. Map of sampling sites. Sampling sites indicated by red pin. 250 *C. maenas* were collected from each site.

PCR and Sequencing

DNA was extracted from all 500 ethanol fixed samples and diluted to 100ng/μl so that they could be screened for the parasite by Polymerase Chain Reaction.

Approximately 25mg of tissue was lysed in a solution of 180 μl buffer ATL and 20 μl proteinase K using a Qiagen TissueLyser II. The rest of the extraction was done using a Qiagen DNeasy Blood & Tissue Kit. Primers MF1 (5'- CCGGAGAGGGGAGCCTGAGA-3') and MR1 (5'-GACGGGCGGTGTGTACAAA-3') as described by Tourtip et al. (2009), were used to amplify a 939 bp fragment of the microsporidian SSU rRNA gene and a 1280 bp fragment of host DNA using a GoTaq flexi PCR reaction (1.25U Taq polymerase, 2 mM MgCl₂, 0.2 mM each dNTP, 1.0 μM each primer, and 0.5 μl of template DNA [100 ng/μl] in a 25 μl reaction volume). Thermocycler conditions were 94 °C for 1 minute, followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min, then a final extension at 72 °C for 10 minutes as described by Bojko et al. (2017). Gel electrophoresis of the PCR products was done through a 1.5% agarose gel at 120 V.

Histology

Formal seawater fixed samples that displayed the pathogen band in PCR were sent to Saffron Scientific Histology Services in Carbondale, Illinois to be mounted onto slides and stained. Each sample was paraffin embedded and sectioned 5 μm thick before being mounted onto two glass slides. One slide was stained with hematoxylin and alcoholic eosin (H&E) and the other with a trichrome stain. The slides were visualized using an Olympus BX51 teaching light microscope and AmScope software was used for imaging.

RESULTS

Sample Analysis

Out of all 500 crabs, only 7 were confirmed to be positive, resulting in a 1.4% prevalence within the green crab population of Maine. Collection site and sex do not appear to have an effect on prevalence. Three positive crabs were collected from Roque Bluffs and four positive crabs were collected from Kettle Cove, but with only seven positive samples, there is no way to be certain if this difference is significant. Similarly, three positive crabs were male and four were female, but this difference can be explained by the fact that the population was 53% female and 47% male. Out of the four females only one was berried and out of all seven crabs only one had a soft shell, so it does not appear that reproduction or shell integrity have any relation to ease of infection. Organ color was described as green-yellow, orange, brown, yellow-brown, yellow, and pale yellow for the seven infected crabs. These descriptions are not different from the rest of the sample and do not suggest that the hepatopancreas of an infected individual outwardly appears distinctly different from that of a healthy crab. Figure 4 below shows that the size distribution of the sample was skewed right. The carapace widths of the entire sample ranged from 21-84 mm with an average of 46.0 mm. The positive crabs had a smaller range of 36-58 mm, and a smaller average carapace width at 44.7 mm. A chi-square analysis was done to examine the difference in mean carapace width, which returned a p-value of 0.85. At the 0.05 significance level this is not a significant difference.

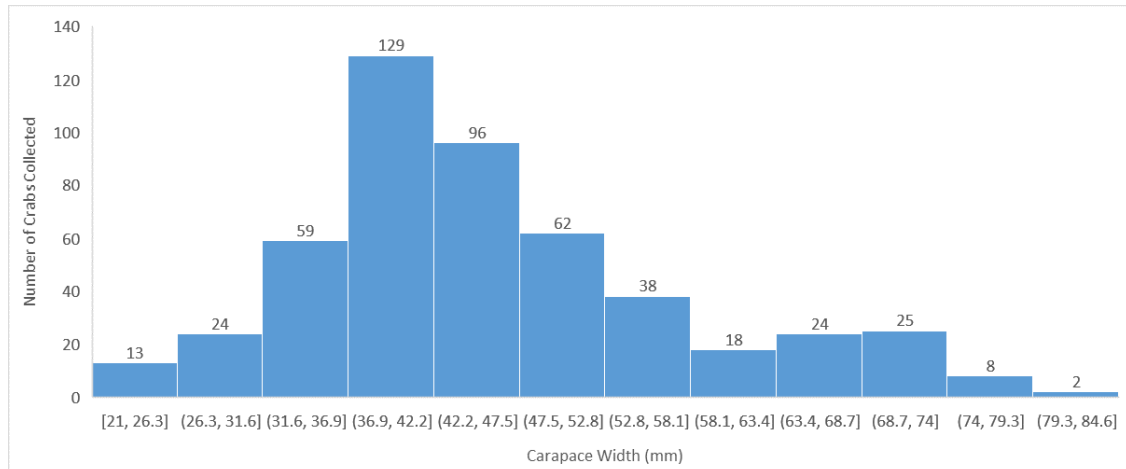


Figure 4. Histogram showing the distribution of carapace widths of the sample.

It also appears that collection date had an effect on prevalence in this sample. As shown below in figure 5, all positive crabs were collected in just three collection days: 7/10, 7/15, and 7/25. The highest number of infected crabs (4 crabs) were collected on 7/10, followed by 7/15 (3 crabs), and 7/25 (1 crab). The number of infected crabs collected appears to peak at 7/10, then gradually decline. Figure 6 shows that the highest number of crabs were also collected on these three days, which could be the cause for the increased prevalence during this time span. However, the most crabs were collected on 7/15, followed by 7/25 and 7/10, so it does not appear that number of crabs collected is directly correlated with the number of infected crabs collected.

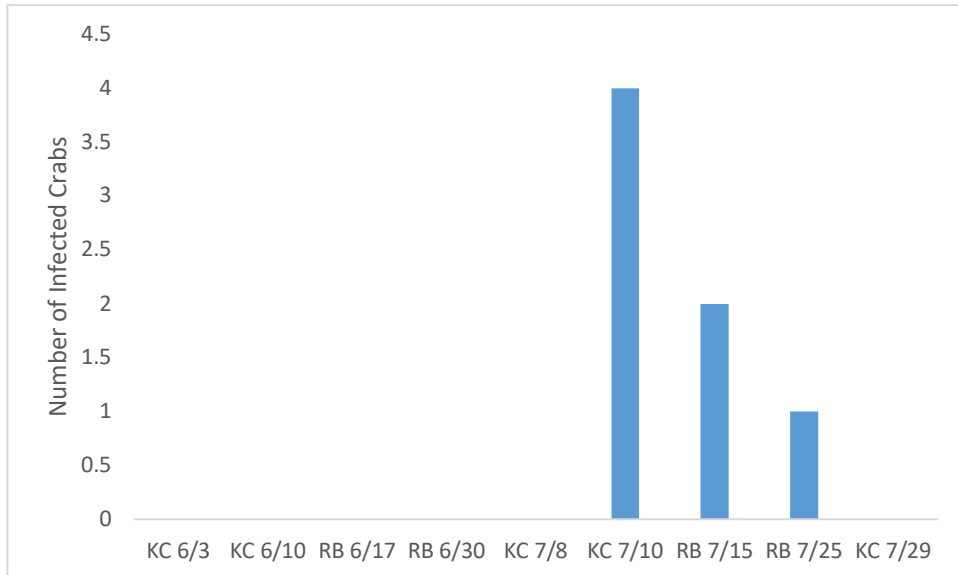


Figure 5. Number of *C. maenas* collected on each day that were later shown to be positive for *Parahepatospora carcini*. All dates are from the year 2019. KC = Kettle Cove, RB = Roque Bluffs. Sample sizes vary for each day.

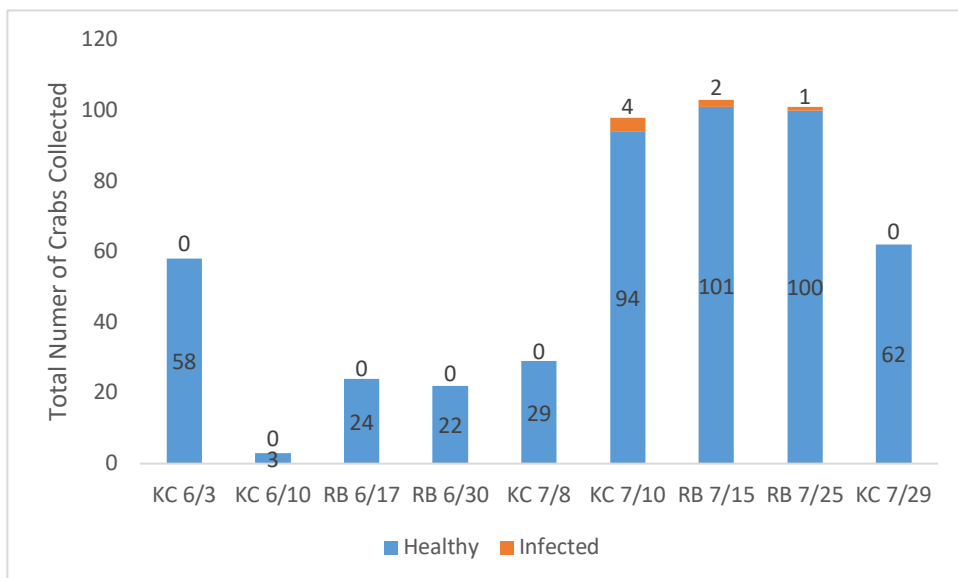


Figure 6. Number of *C. maenas* collected on each day that were later shown to be healthy or infected with *Parahepatospora carcini*. Labels on bars show the number of healthy individuals. Labels over bars show the number of infected individuals. All dates are from the year 2019. KC = Kettle Cove, RB = Roque Bluffs.

PCR and Sequencing

As shown below in figure 7a, seven samples presented a microsporidian band at 939 bp. Because a positive control was not available, the first band to appear around 939 bp in sample number 164 was excised from the gel and DNA was extracted using a Qiagen QIAquick Gel Extraction kit. The DNA was then sent to the University of Maine DNA Sequencing Facility and analyzed using Sequence Scanner 2.0 software. The sequence was confirmed to be from a microsporidian by running the sequence through NCBI GenBank to find a match with another microsporidian. The strongest match was a 94% match with an 18S rRNA gene from the microsporidian *Chytridiopsis typographi*. This sample was then used as a positive control for all other PCR and gel electrophoresis. Before this positive sample was found, the host band at 1280 bp on each sample was used to ensure that the PCR had been successful. Figure 7b shows the results of a PCR reaction on a group of uninfected crabs.

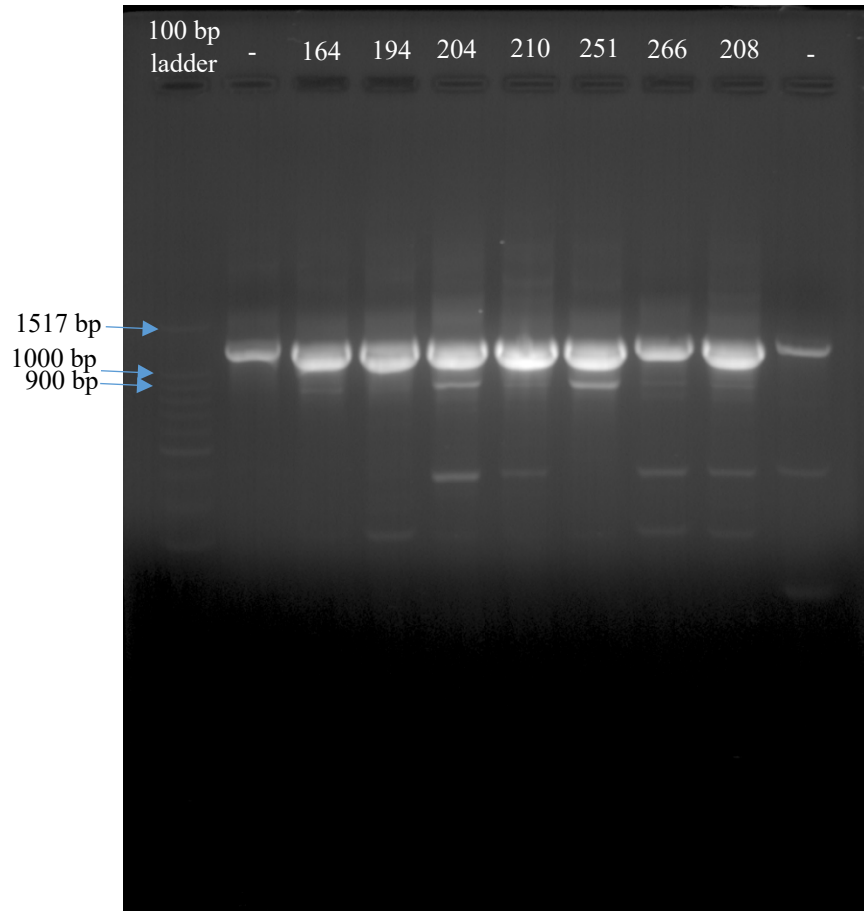


Figure 7a. Gel image of all seven positive samples and two negative samples. Sample numbers of positive samples labelled above lanes. 100 bp DNA ladder labelled in left-most column. (-) indicates non-infected individual

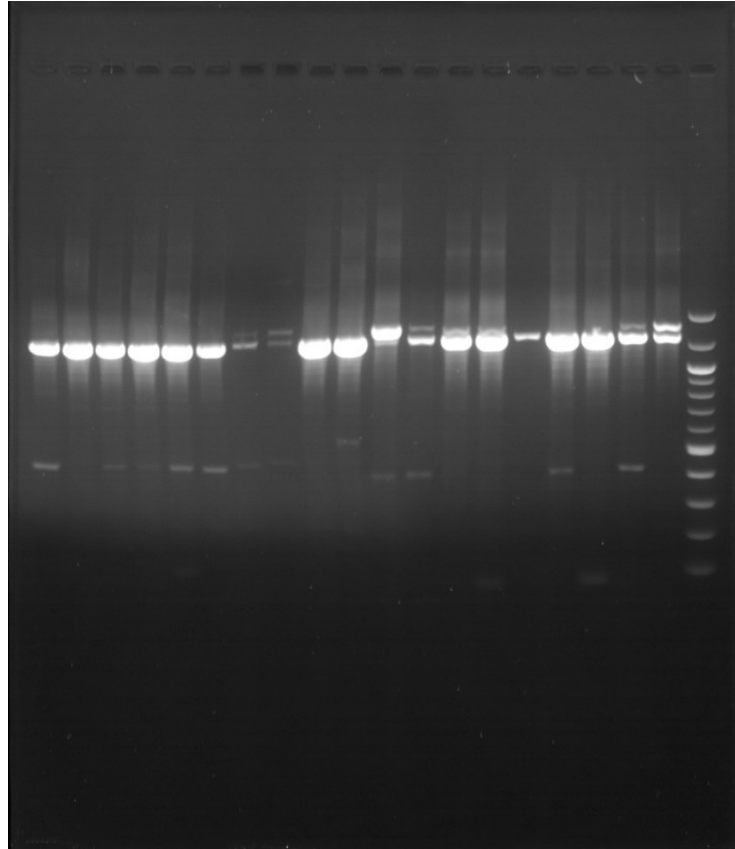


Figure 7b. Gel image of samples 21-39. 100 bp DNA ladder labelled in right-most column. None of these samples expressed a pathogen band at 939bp.

Histology

As shown below in figure 8, histology of the positive samples from PCR confirmed the presence of *Parahepatospora carcini* in all seven crabs. The black arrows in the images point to the spores in each cell. On all slides varying degrees of structural damage from the spores was evident. Spores in earlier life stages remained within the cytoplasm of the infected hepatopancreatic cell. As the spores develop and multiply, they appear to congregate in the tissue nearest the lumen until they eventually lyse the cell and are expelled into the lumen. Once *P. carcini* has broken into the lumen the cell structure is severely damaged and there appears to be very little integrity left within the lumen.

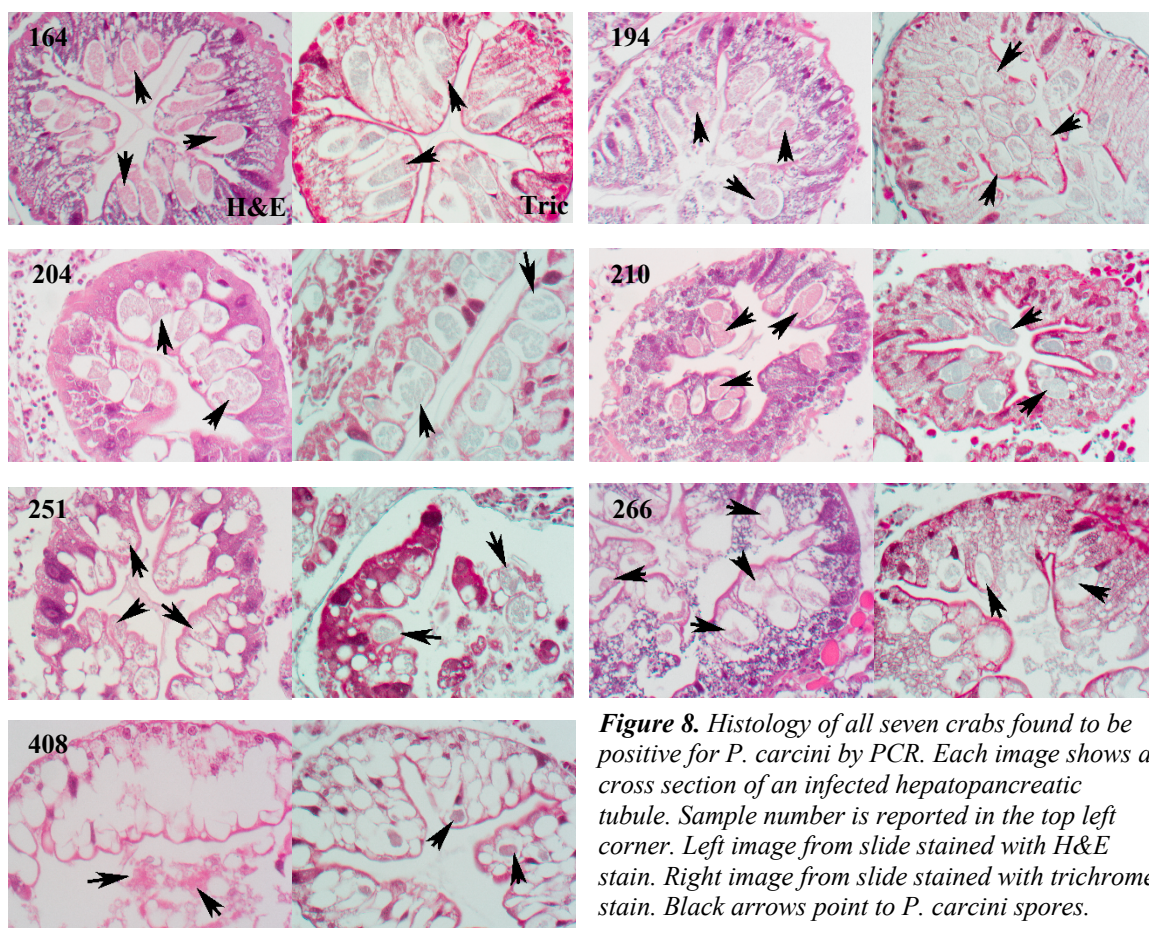


Figure 8. Histology of all seven crabs found to be positive for *P. carcini* by PCR. Each image shows a cross section of an infected hepatopancreatic tubule. Sample number is reported in the top left corner. Left image from slide stained with H&E stain. Right image from slide stained with trichrome stain. Black arrows point to *P. carcini* spores.

In the above figure (8), two different stains are used to examine each figure for *P. carcini* infection. The slide on the left has been stained with H&E stain that is commonly used in histology. The tissue of the hepatopancreas appears purple in color, while *P. carcini* is stained a bright pink. The slides on the right have been stained with a trichrome stain. The host hepatopancreatic tissue is stained a bright pink color and *P. carcini* is left a dull purple. The different affects of the two stains on *P. carcini* and its spores is evident below in figure 9.

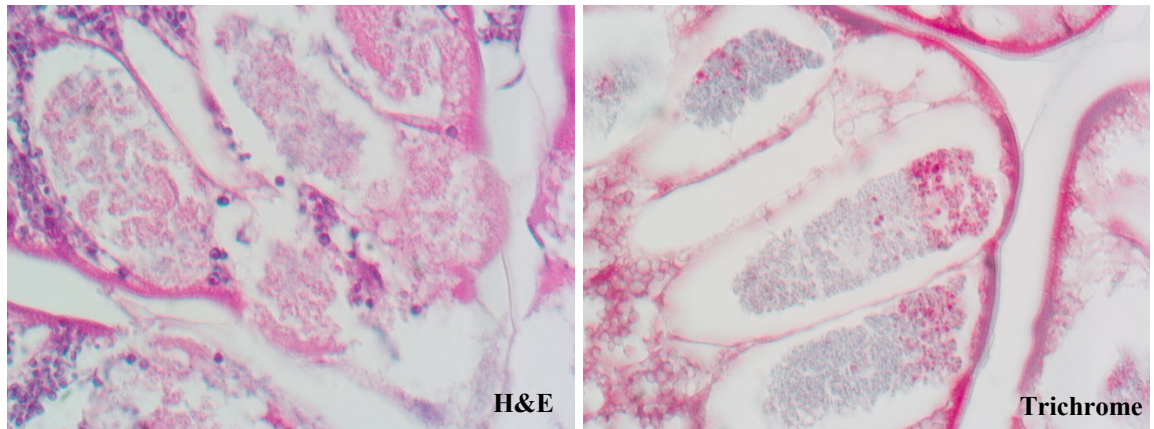


Figure 9. The different effects of an H&E (left) and trichrome (right) stain on *P. carini* and its spores.

DISCUSSION

Although sample location, sex, and organ color appear to have no correlation with *P. carcini* infection, both size and sample date might. The range (36-58 mm) and average carapace width (44.7 mm) of the seven infected crabs were smaller than those of the entire sample (range 21-84 mm, average 46.0 mm). Although the mean carapace width of the infected and uninfected groups was determined not to be significantly different by a chi-square statistical analysis, the infected crab group lacked the larger members of the population that were seen in the uninfected group. The largest crab in the infected group only falls in the 83rd percentile of the entire sample. This could suggest that *C. maenas* does not live long after it has been infected or that infection stunts growth. Since crabs continuously grow as they age, a larger carapace width usually indicates that the crab is older. Therefore, having fewer crabs with larger carapace widths could indicate that the infected crabs are not living to the same age as the rest of the sample. Due to the importance of the hepatopancreas in energy storage during growth phases it could also indicate that the disfunction the parasite causes within the organ impacts growth of the crab.

The sampling data shown in figure 5. shows a pattern for a peak infection period within this sample. The prevalence of *P. carcini* among the entire sample was 1.4%, but among only those three collection days it is 2.3%. The highest prevalence rate was seen on 7/10, when 4.1% of the crabs collected that day were infected with *P. carcini*. After this peak the prevalence decreased over the next two sampling days with a prevalence of 1.9% on 7/15 and 1.0% on 7/25. Based on this data, it appears that *P. carcini* infections peak just before mid-July, then taper off later in the month. If this trend truly represents a

peak infection season it seems strange that there were no infected crabs collected from the same location on 7/8. However, as shown in figure 6 the sample size on this day was much smaller. At the 1.4% prevalence rate, at least 72 crabs would have to be sampled to find one infected crab, but only 29 crabs were collected that day. In fact, on all other sample days the number of crabs collected was fewer than 72. This could have unintentionally created a confounding variable in the data set. To investigate this, a larger, year-round study must be done which not only samples an adequate total number of crabs, but controls the number of crabs collected on each sample day.

Although PCRs were run without a positive control, measures were in place to ensure that all reactions were successful. The presence of a host band from each sample at 1280 bp showed that the PCR was taking place before a positive sample was identified. This is because the selected primers amplified a fragment of *C. maenas* DNA as well as the microsporidia. Therefore, as long as the DNA is from a green crab this band will show up. To ensure that this band was in fact from the host, a band at 1280 bp was excised from a gel and DNA was extracted so that it could be sequenced. The resulting sequence was confirmed to be from the host by comparing the extracted sequence to the *C. maenas* sequence in NCBI GenBank and returning a 99% match. Once a suspected microsporidian band was found, the correct protocols were followed to ensure that all bands that appeared at 939 bp were true positives. Sample number 164 was the first to show a band at the correct size. After gel electrophoresis the band was excised from the gel, purified, sequenced, and compared to NCBI GenBank microsporidian sequences as previously mentioned. The strongest match was a 94% match with an 18S rRNA gene from the microsporidian *Chytridiopsis typographyi*, which is an excellent match because

the 939 bp fragment is also from an 18S rRNA gene of *P. carcini*. After this confirmation it was acceptable to use sample 164 as a positive control for the rest of the PCRs and gel electrophoresis. Any sample that produced a band of the same size was considered to be positive for *P. carcini* because sequencing confirmed that when a microsporidian is present, the primers used produce a band of that size.

For further confirmation histology was performed on all positive samples. Two stains were used to ensure that *P. carcini* was adequately observed and clearly identified. H&E staining is the most common staining system and as such is considered a “standard” for histology. This stained the tissue of the hepatopancreas a deep purple and *P. carcini* a bright pink. To the trained eye it is not difficult to spot the spores, but the difference between the two organisms is not obvious. For this reason, a second set of slides was stained with a trichrome stain. This stain creates a greater contrast between tissue and microsporidian spores, making them easier to identify. The host hepatopancreatic tissue is stained a bright pink color and *P. carcini* is left a dull purple. Using this stain, even the untrained eye could recognize the presence of two different tissues.

CONCLUSION

Using PCR and histology seven out of 500 sampled crabs were confirmed to be infected with *P. carcini*. This results in a 1.4% prevalence of *P. carcini* within the *C. maenas* population of Maine. Sample location, crab sex, and organ color had no correlation with *P. carcini* infection. The seven infected crabs had a smaller range and average carapace width than the entire sample, but the difference in mean carapace width of infected and uninfected crabs was not significant. The absence of larger crabs among the infected group could suggest that once infected, *C. maenas* does not survive for very long or growth is stunted by the damage to the hepatopancreas. *P. carcini* infections in this sample peaked just before mid-July, then taper off later in the month. All seven samples presented a microsporidian band at 939 bp, which was confirmed with DNA sequencing of an excised band. *P. carcini* infection was then verified in each sample with histology using both H&E and trichrome staining techniques. All data and results confirm that *P. carcini* is present in the green crab population of Maine, the first place it has been recorded in the United States.

Should this study be continued in situ hybridization could be used to show the expression of microsporidian 18S rRNA and more conclusively identify *P. carcini* within the tissues. A larger sample must be collected to determine if growth is impacted by *P. carcini* infection and a year-round study would be necessary to investigate how prevalence changes throughout the year. *C. maenas* populations from different states and countries should also be sampled to identify where *P. carcini* originated and how far it has spread. Other native aquatic crustaceans should also be sampled to determine if *P. carcini* can infect other animals with a hepatopancreas.

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AUTHOR'S BIOGRAPHY

I am the only child of Frank and Lisa Torchia. I grew up in the small rural town of Griswold, Connecticut where I attended Griswold High School. I am graduating from the University of Maine with a B.S. in marine science and a minor in animal science. In the fall I will be enrolled in Cornell University's College of Veterinary Medicine. My mother sparked my interest in aquatic animals by taking me to aquariums and zoos as a child. In my free time I enjoy running, hiking, baking, and spending time with animals.